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BONE MORPHOGENETIC PROTEIN-2 STIMULATES ALKALINE PHOSPHATASE ACTIVITY AND COLLAGEN SYNTHESIS IN CULTURED OSTEOBLASTIC CELLS, MC3T3-E1

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SUMMARY: The activities of three bone morphogenetic proteins (BMPs), BMP-1, BMP-2 and BMP-3, on alkaline phosphatase activity, collagen synthesis and DNA synthesis were studied in cultured osteoblastic cells, MC3T3-E1. Treatment of cells with BMP-2 for 48 h induces an increase in cellular alkaline phosphatase activity. This stimulatory effect is evident at a concentration as low as 20 ng/ml of BMP-2 and becomes greater with increasing doses of BMP-2. The BMP-2-induced increase in alkaline phosphatase activity is enhanced by the presence of ß-estradiol, dexamethasone or 1α, 25$(OH)_2D_3$. BMP-2 and BMP-3 slightly but significantly stimulate collagen synthesis. None of the BMPs stimulates DNA synthesis in MC3T3-E1 cells at doses tested. These results indicate that BMPs act directly on osteoblastic cells and stimulate the expression of the osteoblastic phenotypes.

The presence of osteoinductive factors in the bone tissue has long been suggested by the observations that the subcutaneous implantation of demineralized bone matrix induces the process of enchondoral ossification in rat and mouse (1-3). Recently, Wozney et al. have reported the molecular cloning of four such proteins with cartilage and bone-inducing activities, named bone morphogenetic proteins (BMPs) (4). Among the four BMPs, which include BMP-1, BMP-2, BMP-3 and BMP-4, BMP-2, -3 and -4 belong to the transforming growth factor ß (TGFß) supergene family, while BMP-1 has a distinct primary amino acid sequence (4). Recombinant human BMPs have been demonstrated to induce cartilage and bone at implanted sites in rat (4,5). However, the mechanisms of action of BMPs is not understood. In the present

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study we examined possible direct actions of BMP-1, BMP-2 and BMP-3 on cultured osteoblastic cells derived from mouse calvariae, and compared those with the effects of TGF-ß.

Materials and Methods

Cells and materials. MC3T3-E1 cells were grown in phenol red-free α modification of Eagle's minimal essential medium (α-MEM) supplemented with 10% fetal calf serum (MA Bioproducts, Walkersville, MA), 100 μg/ml of streptomycin (Wako, Tokyo, Japan) and 100 U/ml of penicillin G (Meiji Seika, Tokyo, Japan). For experimental purposes, the cells were grown to confluence and serum-deprived by culturing in α-MEM containing 0.3% bovine serum albumin (BSA) for 24 h before experiments. In some experiments, the serum-free conditioned media from CHO cells transfected with either human BMP-1, BMP-2 or BMP-3 cDNA was employed as sources of BMPs (4,5). The concentration of the BMP in conditioned media was ~1 μg/ml, 0.3 μg/ml and 0.01 μg/ml for BMP-1, -2 and -3, respectively, as estimated with the immunoblotting technique. The conditioned medium from non-transfected cells was used as control medium. In other experiments, BMP-2 purified from the serum-free conditioned media (>90% pure) (5) was employed. $1\alpha,25(OH)_2D_3$ was a gift from Roche (Kamakura, Japan). Human TGF-$ß_1$ was obtained from R & D system (Minneapolis, USA). Dexamethasone and ß-estradiol were obtained from Sigma (St. Louis, USA).

Measurements of DNA synthesis, collagen synthesis and cellular alkaline phosphatase activity. The rate of DNA synthesis was determined by measuring incorporation of [^{3}H]thymidine into cellular DNA (7). The rate of collagen synthesis was determined by measuring incorporation of [^{3}H]-proline into collagenase-digestible proteins as described (6). Cellular alkaline phosphatase activity was measured with p-nitrophenyl phosphate as substrate as described (7). Cellular DNA content was measured by the method of Labarca and Paigen (8).

Results

We examined the effects of BMP-1, BMP-2, BMP-3 and TGF-$ß_1$ on DNA synthesis, collagen synthesis and alkaline phosphatase activity in MC3T3-E1 cells (Table 1). None of the three BMPs by itself stimulates DNA synthesis at up to 3% of the concentration of the conditioned media. In contrast, 10^{-10} M of TGF-$ß_1$ inhibits DNA synthesis by 32%. BMP-2 and BMP-3, but not BMP-1, stimulate collagen synthesis by 60 to 70% at 3% of the concentration. TGF-$ß_1$ exerts much more potent stimulatory effect on collagen synthesis. BMP-2 induces a significant increase in cellular alkaline phosphatase activity, but neither BMP-1 nor BMP-3 shows such a stimulatory effect on alkaline phosphatase activity activity at doses tested. In sharp contrast, TGF-$ß_1$ has a profound inhibitory effect on alkaline phosphatase activity as reported previously (9).

Fig. 1 shows the time- and dose-dependent effects of purified BMP-2 on cellular alkaline phosphatase activity. In control cells cellular alkaline

Table 1. Effects of BMPs and TGF-β_1 on DNA synthesis, collagen synthesis and alkaline phosphatase activity in MC3T3-E1 cells

Additions	DNA synthesis	collagen synthesis	alkaline phosphatase activity
	(percent of control)		
None	100±5	100±16	100±7
BMP-1	91±9	98±6	89±9
BMP-2	107±3	159±13*	132±9**
BMP-3	106±3	168±15*	96±24
TGF-β_1	68±1**	337±16**	37±5**

The cells were treated for 24 h (DNA synthesis and collagen synthesis) or 48 h (alkaline phosphatase activity) with the conditioned media containing either BMP-1, BMP-2 or BMP-3 (a final concentration of 3% (v/v)), the condition media from mock transfection (3%) or TGF-β_1 (10^{-10} M). Control (none) values are 27000±1300 cpm/well for DNA synthesis, 2270±370 cpm/well for collagen synthesis and 11.4±0.5 nmol/min/g protein for alkaline phosphatase activity. Values are meas±SD of three or four determinations. Asterisks represent statistically significant differences from controls (none) values (*: $P<0.05$; **: $P<0.01$).

phosphatase activity declines significantly after 48 h. By contrast, in cells treated with BMP-2 alkaline phosphatase activity rises remarkably after 48 h and stays at this level for at least the next 24 h. The stimulatory effect of BMP-2 is observed at concentrations as low as 20 ng/ml and increases with

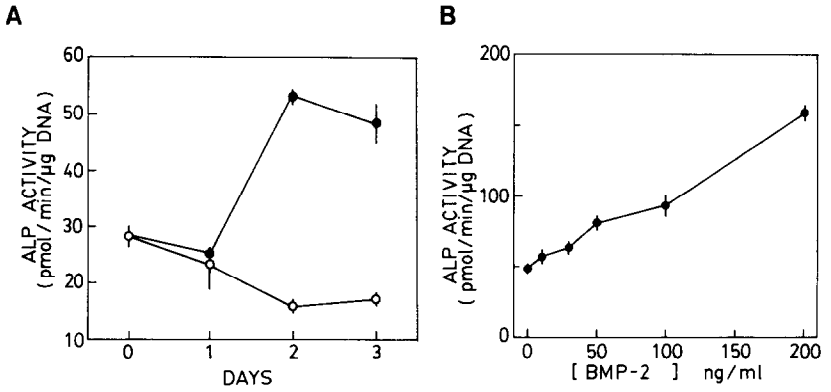

Fig. 1. Time- and dose-dependent effects of purified BMP-2 on cellular alkaline phosphatase activity. A. The cells were cultured in α MEM containing 0.3% BSA with (●) or without (○) BMP-2 (100 ng/ml) for indicated time periods. B. The cells were treated with various concentrations of BMP-2 for 48 h. Values are means±SD of four determinations.

Table 2. Effects of ß-estradiol, dexamethasone and 1α,25(OH)$_2$D$_3$ on purified BMP-2-induced increases in alkaline phosphatase activity in MC3T3-E1 cells

Additions	Alkaline phosphatase activity pmol/min/µg DNA	
None	83±3	(100%)
BMP-2A 100ng/ml	196±10	(236%)**
ß-Estradiol 10^{-8} M	84±6	(101%)
Dexamethasone 10^{-8} M	88±3	(106%)
1α,25(OH)$_2$D$_3$ 10^{-10} M	118±9	(142%)**, §
BMP-2A+ß-Estradiol	232±6	(280%)**, §
BMP-2A+Dexamethasone	299±18	(360%)**, §§
BMP-2A+1α,25(OH)$_2$D$_3$	320±16	(386%)**, §§

The cells were treated with various agents for 48 h. Values are means±SD of four determinations. Symbols (* and §) represent statistically significant differences compared to control (none) values (**) or values in BMP-2-treated cells (§ and §§) (§: $P<0.05$, ** and §§: $P<0.01$).

increasing doses of BMP-2 up to 200 ng/ml. This effect of BMP-2 requires more than 24 h: BMP-2 does not stimulate cellular alkaline phosphatase activity at 24 h.

As shown in Table 2, either ß-estradiol (10^{-8} M) or dexamethasone (10^{-8} M) alone does not significantly stimulate alkaline phosphatase activity. As reported previously, 1α,25(OH)$_2$D$_3$ (10^{-10} M) by itself slightly but significantly stimulates alkaline phosphatase activity (10). When BMP-2 (100 ng/ml) is added together with each of these sterols, the induction by BMP-2 of alkaline phosphatase activity is significantly enhanced.

Discussion

The results in the present study demonstrate for the first time that human BMPs exert direct effects on osteoblastic MC3T3-E1 cells to induce expression of the differentiated phenotypes of bone cells: alkaline phosphatase activity and collagen synthesis. Among BMP-1, -2 and -3, BMP-2

induces increases in both the cellular alkaline phosphatase activity and the rate of collagen synthesis (Table 1 and Fig. 1), whereas BMP-3 has a stimulatory effect only on collagen synthesis (Table 1), and BMP-1 shows little effect, if any, on either parameter, at doses tested in the pressent study. Vukicevic et al. (11) very recently reported that osteogenin, a bone-inductive protein, which was isolated from bovine bone and thought to be identical to BMP-3, stimulated both alkaline phosphatase activity and the rate of collagen synthesis in the primary culture of rat calvarial osteoblasts. Although the reason for the discrepant results is not clear, the differences in the source (recombinant vs purified from bovine bone), species or doses of BMP and cell types employed in the experiments may be responsible. It is notable that both BMP-2 and BMP-3 individually have significant influences on the expression of the osteoblastic phenotypes under the serum-free condition of culture. These results suggest that the osteoinductive effect of BMPs is at least in part due to the direct actions on cells of the osteoblastic lineage. However, it is also possible that BMP-2 exerts its effect indirectly via the production of another growth factor, which may alone or in combination with BMP-2 act on MC3T3-E1 cells to enhance alkaline phosphatase activity. The lag time longer than 24 h may support the latter possibility. TGF-β_1, which has significant structural similarities to BMP-2 and BMP-3, potently stimulates collagen synthesis like BMP-2 and -3. However, differently from these BMPs, TGF-β_1 inhibits alkaline phosphatase activity and DNA synthesis (Table 1). These findings suggest distinct and unique regulatory activities of BMPs in bone metabolism.

As shown in Table 2, the concomitant presence of the bioactive sterols with known modulatory effects on osteoblastic functions enhances BMP-2-induced increases in cellular alkaline phosphatase activity. These results suggest that the complex interactions of the BMPs with these sterol hormones and other peptide growth factors, such as TGF-ß, platelet-derived growth factor and fibroblast growth factor, may be important for the regulation of development and repair of bone in vivo (12).

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